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GENETIC ENGINEERING OF CLOSTRIDIUM DIFFICILE TOXIN A VACCINE

ANNUAL/FINAL REPORT

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FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

The investigator(s) have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1992) and the Administration Practices Supplements.

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SUMMARY

Recombinant DNA technology allows for an approach to subunit vaccine production that should provide advantages over existing techniques. Improvement of vaccine biotechnology in the area of recombinant DNA studies using *Clostridium difficile* toxin A as the model, is the major long range objective of this project.

A genomic library of λ gt11 of *C. difficile* chromosomal DNA was screened using anti-toxin A which resulted in the identification of one stable positive clone, λ cd19. The insert in λ cd19 was demonstrated to be a 0.3 kb fragment by restriction digestion, and by hybridization of the clone to a chromosomal digest of *C. difficile*. Verification of the immunological identity of the isolated toxin A gene fragment in λ cd19 was determined by affinity purifying toxin A antibodies specific for λ cd19 gene product, and using these selected antibodies to probe a Western blot of purified toxin A. After further biological characterization of this clone, it was determined that this fragment contains an important antigenic region. The DNA of this fragment has been sequenced, and the amino acid sequence deduced.

This 0.3 kb toxin A positive fragment was demonstrated by Southern blot analysis to hybridize to a 16 kb fragment from a HindIII digestion of *C. difficile* chromosomal DNA. In an attempt to isolate the entire toxin A gene, the 16 kb HindIII fragment was cloned into λ 2001. The HindIII fragment was cloned by digesting *C. difficile* DNA with HindIII, and separating the DNA fragments in the range of 12 to 20 kb using high pressure liquid chromatography. These restriction fragments were then ligated into the HindIII site of λ 2001, and packaged in *E. coli* NM539. The recombinant phages were screened with a synthetic 40 bp probe which was derived for the sequence of the 0.3 kb fragment. A λ 2001 recombinant which hybridized to the toxin A probe has been isolated and partially characterized.

To fulfill the contractual arrangement which was to assist in the development of the area of vaccine biotechnology using recombinant DNA techniques, and *C. difficile* toxin A as the model, a unique series of experiments were used. In summary, a suspected immunogenic fragment of *C. difficile* toxin A was PCR amplified, subcloned into expression vectors, and tested for antigenicity using antibodies made against toxin A. Upon identifying fragments that are immunoreactive, a series of overlapping exonuclease III generated deletion mutants were produced and subcloned in the BlueScript expression vector. Selected N- and C-termini truncated fusion proteins generated from the deletion mutants were tested by Western blotting to determine which mutants retained antigenic determinants.

BACKGROUND

Recombinant DNA technology allows for an approach to subunit vaccine production that should provide advantages over existing techniques. The major long range objective of this project is directed toward the improvement of vaccine biotechnology in the area of recombinant DNA studies using *Clostridium difficile* toxin A as the model. This was proposed to be accomplished by using a unique series of recombinant DNA techniques to map epitopes.

1. *C. difficile* Toxin A Literature Review

C. difficile was first described in 1935 (1), but has only been recognized as a clinically significant pathogen within the last twelve years. This toxin producing nosocomial pathogen (2,3,4,5,6) is the causative agent for the diarrheal syndrome termed antibiotic associated pseudomembranous colitis (PMC) (7,8,9,10). PMC is a disease of the lower gastrointestinal tract that can be histopathologically characterized by exudative plaques on the bowel mucosa. If left untreated, it can be fatal. The etiology of PMC does not only depend of colonization of *C. difficile* toxin producing strains, but other factors affecting the gastrointestinal tract may initiate the disease state, such as surgery, cancer chemotherapy and most frequently antibiotic therapy (11,12,13,14,15).

Pathogenicity and cytotoxicity associated with PMC has been linked to production of two toxins, A (enterotoxin) and B (cytotoxin). There have been a number of publications describing biological characteristics of toxins A and B (16,17,18,19,20,21,22) and evidence has developed to indicate that toxins A and B work synergistically to cause PMC (23,24).

Toxin A is a large protein that has been demonstrated to elicit a hemorrhagic fluid response in the rabbit intestinal loop assay, cause fluid accumulation in the suckling mouse assay (25,26), exhibit cytotoxic activity on mammalian tissue culture cells (27), and bind to and agglutinate rabbit erythrocytes (28). The molecular weight of toxin A has been reported by several different groups, using non-denaturing gels and gel filtration, to be approximately 440,000 to 600,000 (29,30,31). Studies directed toward the molecular genetics or DNA of *C. difficile* toxin A have been appearing in the literature in increasing numbers in the last several years. The first paper published on cloning part of *C. difficile* toxin A was reported by our group (32). Since this publication, several other studies have followed on cloning, expression of *E. coli*, and sequencing of toxin A and B (33,34,35,36,37,38). We have also recently published a paper that demonstrates that toxic strains of *C. difficile* normally contain the genetic composition for toxin A and B simultaneously (39).

The objective of this project is to use toxin A of *C. difficile* in a unique series of molecular procedures for the prediction, identification and purification of antigenic sites, which may ultimately be used as a model system for the development of subunit vaccines. Recombinant DNA technology allows for an approach to subunit vaccine production that should provide advantages over existing techniques. Potential advantages of genetically engineered antigens for the preparation of vaccines are stability, purity, safety of preparation, price, lack of side effects and variety of serotypes. A number of determinants coding for antigens have been cloned from viruses, bacteria, parasites and toxins, with the long range goal of producing better vaccines and improving molecular techniques for the development of vaccines. Improvement of vaccine biotechnology in the area of recombinant DNA research is the major long range objective of this project.

MATERIALS AND METHODS

1. Affinity Purification of Antibodies

Antibodies specific for the toxin-positive peptide encoded by recombinant λ gt11 phage particles were affinity purified from toxin A antiserum using the procedure described by Lyon et al. (40) with the following variations. Recombinant phage were plated at a density of approximately 6,000 plaques per plate (150 mm diameter). Nitrocellulose filters were overlaid on the agar plates and incubated at 37°C for 16 hours which allowed for binding of antigens. The filters were next removed and suspended overnight in a 1:50 dilution of *E. coli* adsorbed toxin A antiserum. After washing the nitrocellulose paper twice in TTBS (0.05% tween 20, 50 mM tris, 150 mM NaCl, pH 8.0) and once in 10 ml saline, specific antibodies which bound to the nitrocellulose filters were eluted by adding 10 ml of glycine buffer per filter (0.2 M glycine, 0.15 M NaCl, pH 2.8) for 10 minutes. Next, the nitrocellulose paper was removed from the glycine buffer and the pH of this selected antibody solution was neutralized by adding 8 mg of Tris per ml of eluate.

2. Electrophoresis and Blotting

Non-denaturing polyacrylamide gel electrophoreses (PAGE) of toxin A was performed in a 5 to 15% gradient slab gel at pH 8.3 (41). Gels were stained with Coomassie Blue R-259. Electrophoretic transfer (Western blotting) of proteins from polyacrylamide gels to nitrocellulose paper was performed as described by Towbin et al. (42).

DNA was separated on a 0.8% agarose gel and stained with ethidium bromide (43). Nick translation with [α -³²P] dCTP and Southern blot hybridizations were performed as described in the nick translation and sure blot hybridization kits provided by Oncor, Inc. (Gaithersburg, Maryland, U.S.A.).

3. Cytotoxicity Testing

Supernatant from *C. difficile* cultures, purified or partially purified toxin A, and crude lysates from *E. coli* Y1090 (obtained from Promega Biotec) infected with recombinant λ gt11 (43) and induced with isopropyl thio-beta-D galactopyranoside (IPTG) (44), were filter sterilized and cytotoxic activities determined using 3T3 mouse fibroblasts as described previously (32).

4. DNA Sequence Analysis

Sequence analysis of DNA encoding for toxin A was determined using the Sanger chain-termination sequence procedure from the United States Biochemical Corp. (Cleveland, OH). Protocols and reagents from the TAQuence™ kit (United States Biochemical Corporation, Cleveland, Ohio) were used for high temperature chain termination DNA sequencing to read through compressions.

5. DNA Synthesis

Oligodeoxynucleotides were synthesized using beta-cyanoethyl phosphoramidite chemistry in a MilliGen 7500 DNA synthesizer. Analysis and purification of oligonucleotides were performed by either separating DNA by polyacrylamide gel electrophoresis in 15% to 20% gels, or by fractionating DNA on a trityl-specific reverse phase Delta Pak C18 (Water, Inc.) high pressure liquid chromatography (HPLC) column.

6. Screening Predicted Epitopes

Synthetic DNA oligomer were cloned and screened using the λ gt11 expression system of Young and Davis (44) with the following alterations. The oligomers that were ligated into the EcoRI site of λ gt11 were packaged with PackagenTM (Promega Biotec, Madison, WI), and the recombinants were directly screened without amplification. Toxin A antigen-production plaques were monitored on nitrocellulose filters using a 1:200 dilution of *E. coli*- λ gt11 absorbed anti-toxin A, and the Bio-Rad horseradish peroxidase-bound goat anti-rabbit IgG immunoblot kit (Richmond, CA).

7. Restriction Digestion, Cloning and Screening

Restriction endonuclease, digestion, and enzymatic manipulation of DNA for cloning were performed as described by suppliers. Standard procedures that were used for isolation and manipulation of DNA for cloning (43). Oligomers were radiolabeled by second strain synthesis using [α -³²P] dATP, and DNA hybridizations were performed as described earlier.

8. Computer Analysis

Nucleotide sequence data was analyzed by an IBM PC-XT computer with programs from International Biotechnologies, Inc., and a Digital Microvax computer using the database and graphic programs of Cage/Gem (Battelle, Pacific Northwest Laboratories, Richland, Washington). Epitopes were predicted with the use of Pustell programs from International Biotechnologies, Inc., and the MSEQ programs from the University of Michigan.

9. PCR Amplification

Amplification of a 800 bp *C. difficile* toxin A gene fragment was carried out in a 50 μ l reaction volume containing 50 ng of *C. difficile* DNA, 10mM Tris-HCl (pH 8.3), 50mM KCl, 1.5 mM MgCl₂, 2.5 units of Tag polymerase (Perkin Elmer, Norwalk, CT, USA), 200 μ M dNTP mix, and 0.2 μ M oligonucleotide primers. To ensure that all nuclease activity was destroyed, the reaction tube was heated for 5 minutes at 94°C prior to adding the Tag polymerase. Amplification was conducted for 37 cycles using a Perkin Elmer Cetus thermocycler (Norwalk, CT, USA). The cycle used to amplify the toxin A gene fragment consisted of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 65°C for 1 minute.

RESULTS AND DISCUSSION

1. Toxin A Cloning and Characterization

In the original proposal we reported preliminary screening for toxin A antigen producing recombinants in a λ gt11 genomic library of *C. difficile* DNA. Seven positive clones were identified and reported in this proposal; however, in subsequent studies all but one of the identified positive plaques spontaneously lost the *C. difficile* DNA inserts. This was evident by a revision from clear to blue plaques, loss of immunological reactivity with toxin A antiserum, and by analyzes of DNA inserts on agarose gels. λ cd19 was the only recombinant plaque stable enough to allow for consistent immunological verification; however, this clone reverts from clear to blue plaques at a frequency of 2 percent. (It should be noted that other genes have been screened for in this library and are stable.)

Verification of the immunological identity of the isolated toxin A gene fragment in λ cd19 gene product, and using these selected antibodies to probe a Western blot of purified toxin A (fig. 1). *E. coli* lysates generated from infecting Y1090 with λ cd19 phage particles and induced with IPTG, were negative for cytotoxic activity on 3T3 mammalian tissue culture cells.

The EcoRI cloning site in λ gt11 is contained within a 2.08 kb SacI-KpnI fragment (44); therefore, λ cd19 DNA was double digested with SacI-KpnI. This 2.08 kb SacI-KpnI fragment was increased in size in λ cd19 to 2.40 kb, thereby demonstrating the presence of a 0.3 kb TaqI insert (fig. 2.) (Note, the genomic library was generated with TaqI.) A 0.3 kb *C. difficile* DNA insert in λ cd19 was also demonstrated by hybridizing [α - 32 P] nick translated λ cd19 DNA to a *C. difficile* TaqI chromosomal digest (fig. 3.) The [α - 32 P] labeled cd19 probe also hybridized to a 4.5 kb fragment in a PstI chromosomal digestion of *C. difficile*, and a 16 kb fragment in a HindIII digestion (fig. 3).

The toxin A gene fragment in λ cd19 was not recoverable from EcoRI digestion of this DNA, even though this insert was ligated into the EcoRI site of λ gt11. To determine why this DNA insert did not digest out with EcoRI, the SacI-KpnI fragment was subcloned into M13 (mp18 and mp19) and mapped. The restriction map revealed that a single EcoRI site adjacent to the SacI site in λ cd19 was destroyed or some how protected from digestion following initial cloning (fig. 4). A 1.4 kb EcoRI-SacI fragment containing the 0.3 kb *C. difficile* gene was next subcloned in M13mp18 and M13mp19 for sequencing. Due to the presence of 1.02 kb of λ gt11 lac Z DNA preceding *C. difficile* insert within M13mp18, it was sequenced with a reverse primer, whereas the insert subcloned in M13mp19 was sequenced using M13 universal primer.

To isolate the entire toxin A gene, the 16 kb HindIII *C. difficile* DNA fragment that hybridized to the 0.3 kb toxin A fragment was cloned into λ 2001 (fig. 5.) This fragment was obtained by digesting *C. difficile* chromosomal DNA with HindIII, and separating the DNA fragments in the range of 12 to 20 kb using the Ultrahydrogel (Waters Inc.) DNA HPLC column. These restriction fragments were then ligated into the HindIII site of the replacement vector λ 2001, and packaged into *E. coli* NM539. The recombinant phages were screened with a 32 P 40 base pair synthetic DNA probe derived from the sequence of λ cd19. A λ 2001 recombinant which hybridized to the toxin A probe has been isolated and partially characterized.

Tissue culture cytotoxicity studies conducted on the λ 2001 recombinant indicated that this toxin A clone does not express toxic activity. However, based on preliminary map and hybridization data generated on this recombinant, and results published by Von Eichel-Steiber et al. (35), this 16 kb fragment contains approximately 9 kb of the 10 kb fragment cloned by Von Eichel-Steiber, and contains the 4 kb region that is reported to encode for part of toxin A. The additional *C. difficile* DNA contained in the fragment cloned into λ 2001 may contain a substantial region of the toxin gene.

2. Epitope Prediction and Testing

During the funded period of this contract, a paper was published by C. H. Dove and coworkers (36) on the entire *C. difficile* toxin A gene sequence. Based on the data published by this group, and data published by Von Eichel-Steiber's group (35) on overlapping toxin A DNA fragments which express antigenic sites, the DNA sequence of an 800 bp fragment was identified which is thought to have a major antigenic site. This 800 bp fragment has also been identified to contain the 300 bp fragment that we earlier reported on cloning (32). Using the above information epitopes were predicted using the Antigenic Index of the IBI Pustell software program.

The antigenic index prediction method described by Jameson and Wolf (45) generates values for surface accessibility parameters and combines these values with those obtained for regional backbone flexibility and predicted secondary structure. The linear surface contour profile generated from these calculations allows for predicting potential continuous epitopes. Twelve regions representing the highest Antigenic Index peaks were selected and DNA oligomers for these corresponding regions were synthesized (fig. 6). Additional bases were added to these fragments in order to generate an *Eco*R1 site. These synthetic single strain oligomers were then annealed and ligated into λ gt11 and subsequently tested positive for the generation of clear plaques using X-gel. The clones were then tested with antiserum against toxin A to assay for antigenicity. Under the immunoblot conditions tested peptides produced by these cloned fragments did not react with toxin A antibodies.

The 12 epitopes predicted represent the most probable antigenic regions using the IBI and MESQ software. These predicted and tested regions may not have been antigenic due to a variety of reasons. First, parameters for prediction did not allow for the selection of the appropriate epitopes. Second, the antigenic sites in this 800 bp fragment may represent discontinuous epitopes which cannot be selected using the above described procedures. Third, the antigenic site(s) from this particular system could not be expressed in the lac Z gene of λ gt11. And finally, these AT rich DNA fragments were not stable in the λ gt11 cloning system. This final explanation requires further discussion, because we have demonstrated that *C. difficile* toxin A DNA is highly unstable in λ gt11.

From the original library of *C. difficile* DNA, that was constructed in λ gt11, approximately 35,000 plaques were screened for toxin A antigen-producing clones resulting in the original detection of 22 positive plaques. All but one of the identified positive plaques spontaneously lost *C. difficile* inserts after plaque purification and amplification as indicated by immunologically testing for toxin A cloned antigens, and by revision from clear to blue plaques. λ cd19 (the 300 bp *Taq*I clone) was the only recombinant plaque stable enough to allow for consistent immunological verification; however, as reported above, this clone lost one of the *Eco*R1 cloning sites. It should be emphasized that this 300 bp *Taq*I fragment in λ cd19 truly represents part of the toxin A gene. This has been verified by the numerous references that site the cloning of this fragment, and by the correlation of DNA hybridization patterns (33,34,35,36,46,47).

However, the most convincing data that demonstrates the presence and location of this 300 bp cloned fragment comes from Dove's (36) DNA sequence of toxin A. The sequence data demonstrates that there is only one 300 bp TaqI fragment within the toxin A gene, which is located within a 4.5 PstI fragment, and a large HindIII fragment. Hybridization data published by our laboratory (32) demonstrates that [α -³²P] labeled λ cd19 hybridized to a 4.5 kb fragment in a PstI chromosomal digestion of *C. difficile*, and a 16 kb fragment in a HindIII digestion (fig. 3.)

Despite the apparent stability of λ cd19, it mutated soon after the initial characterization of this clone. It was determined that this clone had significantly mutated after it lost its ability to produce a peptide that would react with toxin A antiserum, and after the DNA sequenced was compared to Dove's sequence (36). Consequently, it can be predicted that one possibility that a positive epitope could not be identified using the predicted cloned oligomers in λ gt11 was due to the fact that *C. difficile* DNA is not stable in λ gt11.

Through personal communication with numerous other investigators that use the λ gt11 cloning system, it has become apparent that AT rich DNA is not stable in λ gt11. *C. difficile* DNA is 70% AT rich.

3. Epitope Mapping By Deletion Mutagenesis/Annual Report June 15, 1990 to June 16, 1991

To fulfill the contractual arrangement which were to assist in the development of the area of vaccine biotechnology using recombinant DNA techniques, and *C. difficile* toxin A as the model, an alternative expression system was used. In summary, a suspected immunogenic fragment of *C. difficile* toxin A was PCR amplified, subcloned into expression vectors, and tested for antigenicity using polyvalent antiserum made against toxin A. Upon identifying fragments that are immunoreactive, a series of overlapping exonuclease III generated deletion mutants were produced and subcloned in the BlueScript expression vector (48,49). Selected N- and C-terminal truncated fusion proteins generated from the deletion mutants were tested by Western blotting to determine which mutants retained antigenic determinants.

To map *C. difficile* toxin A by deletion mutagenesis, it is essential to subdivide this large 8.1kb gene into smaller fragments approximately 1kb length. This was performed by strategically determining, with the appropriate parameters in consideration, primer sequences along the length of the toxin A gene. Primers were identified and a number of them have been synthesized. To date six fragments from toxin A have been PCR amplified, cloned and expressed in the BlueScript vector.

Due to past problems encountered with stability of *C. difficile*'s 70% AT rich genome (as detailed above), it was imperative to determine the stability of the PCR amplified fragments in the BlueScript cloning system. After a series of controlled experiments it was determined that *C. difficile* DNA was stable in this system. This was accomplished by repeatedly subculturing these recombinants over a period of two months, and monitoring the size and the integrity of the expressed peptides.

The six PCR amplified fragments were cloned into BlueScript and screened by immunoblotting for expression of immunogenic peptides or epitopes. Dr. Tracy Wilkins of the Department of Anaerobic Microbiology, Virginia Polytechnic Institute and State University, was kind enough to donate monoclonal antibody PCG-4 which reacts against the carbohydrate-binding portion of toxin A. Only one clone expressed a peptide which was recognized by the monoclonal. This 300 bp clone overlapped the 0.3 kb fragment

described above. This finding is consistent with our initial supposition that the 0.3kb fragment which was cloned and sequenced contained the carbohydrate-binding region or the receptor site.

RECOMMENDATIONS AND CONCLUSIONS

One of the main objectives of this contract is to develop a system for testing epitopes using expression vectors such as λ gt11, which includes reporting potential problems and solutions to these problems. From our experience of cloning toxin A, and as well as toxin B gene fragments in λ gt11, and from personal communications with other investigators, we have identified a non-published common problem encountered when using this cloning system. AT rich DNA is not stable in λ gt11. This cloning system as well as cosmid cloning systems should be avoided when the GC content of the DNA to be cloned is not similar to E. coli. By surveying the literature it appears that pBR322 derived vectors (including expression vectors) are more stable when using AT rich DNA.

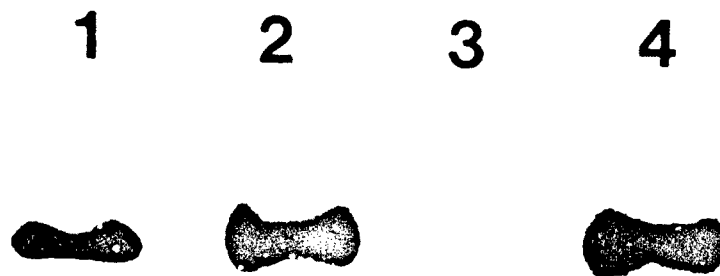


Figure 1. Purity of toxin A, and confirmation of cloned toxin determinants. PAGE and Western blot analysis were performed as described in materials and methods. Lanes: (1) PAGE of 6 ug purified toxin A. (2) Western blot of toxin A using control toxin A antiserum, 1:200 dilution. (3) Western blot of toxin A with selected antibodies from lambda cell control plaques. (4) Western blot of toxin A with antibodies selected from lambda cd19.

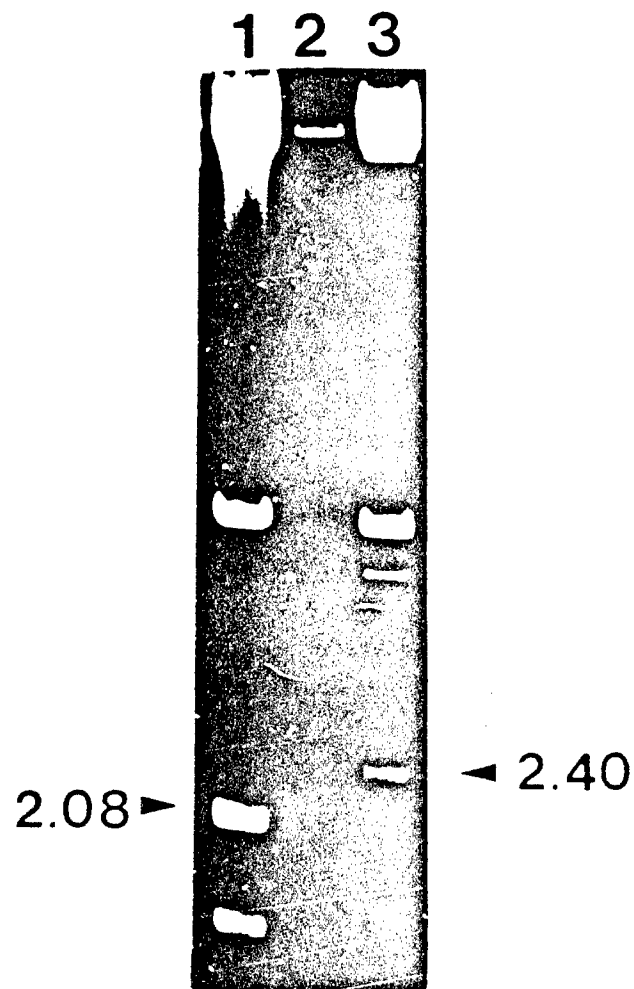


Figure 2. Restriction digestion of lambda gt11 and lambda cd19. Lambda gt11 (lane 1) and lambda cd19 (lane 3) were double digested with SacI and KpnI as described by supplier. The band immediately above the 2.4 kb fragment in lane 3 represents incomplete digestion of the 2.4 kb fragment and the lower migrating 1.51 kb fragment. Lane 2 contains the molecular weight standard HindIII digested lambda DNA (BRL Inc., Gaithersburg, Maryland USA).

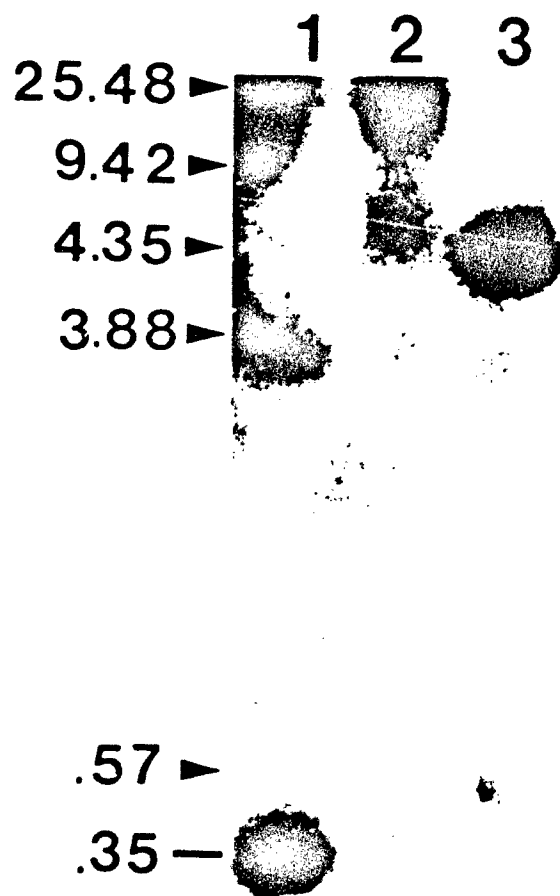


Figure 3. Southern hybridization of restriction digested *C. difficile* chromosomal DNA with [α - 32 P] dCTP labeled lambda cdl9. *C. difficile* chromosomal DNA was digested with TaqI, lane 1; HindIII, lane 2; and PstI, lane 3.

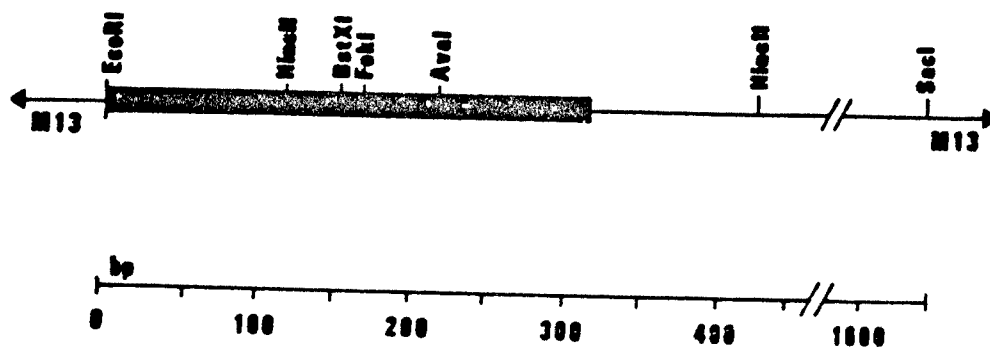


Figure 4. Restriction map of λ cd19mp19. An EcoRI-SacI fragment from λ cd19 containing the 0.3 kb toxin A insert was cloned in to M13mp19. The thick bar represents the toxin A insert.

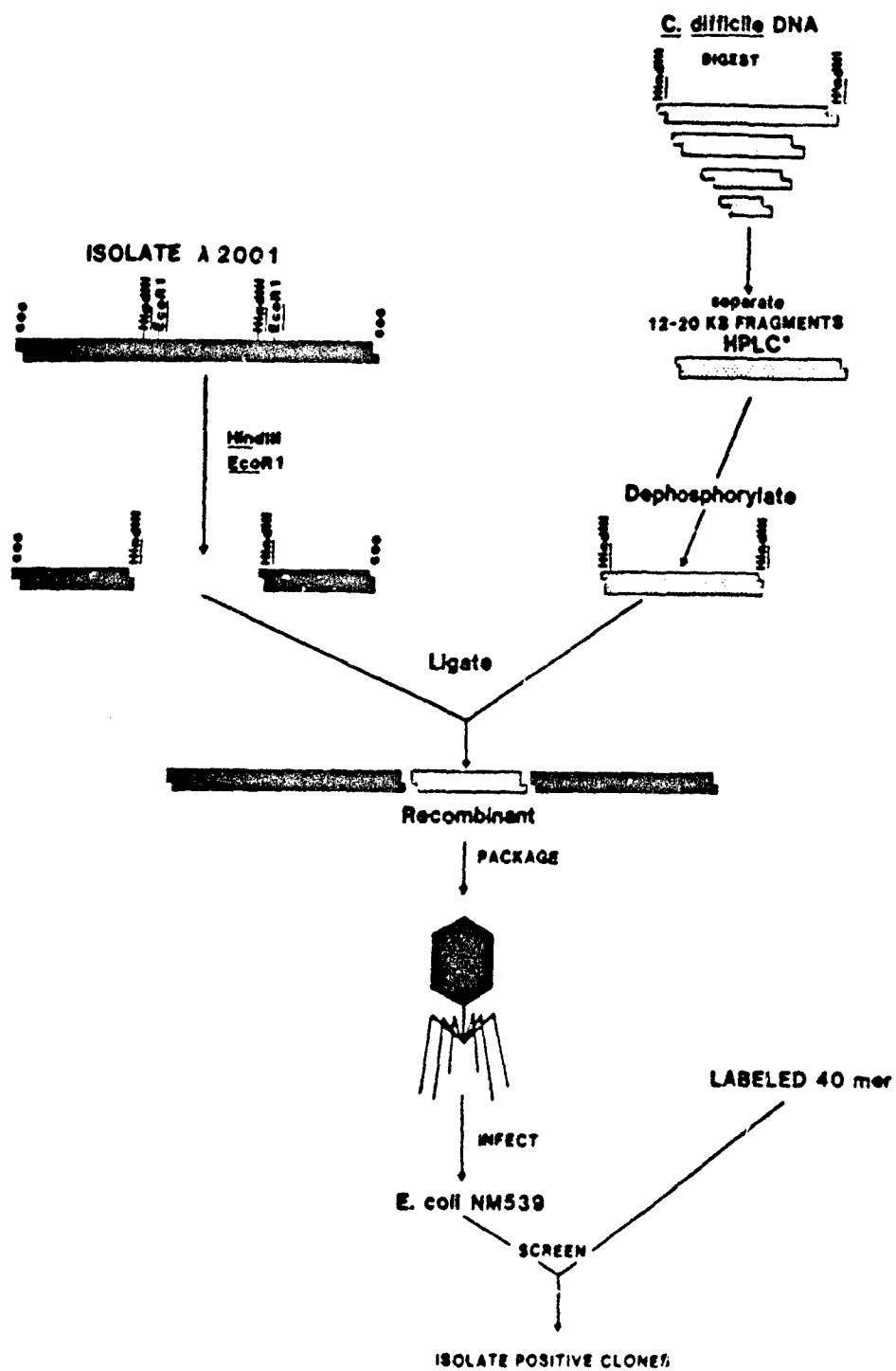


Figure 5. Cloning scheme for the 16 kb *Hind*III toxin A restriction fragment.

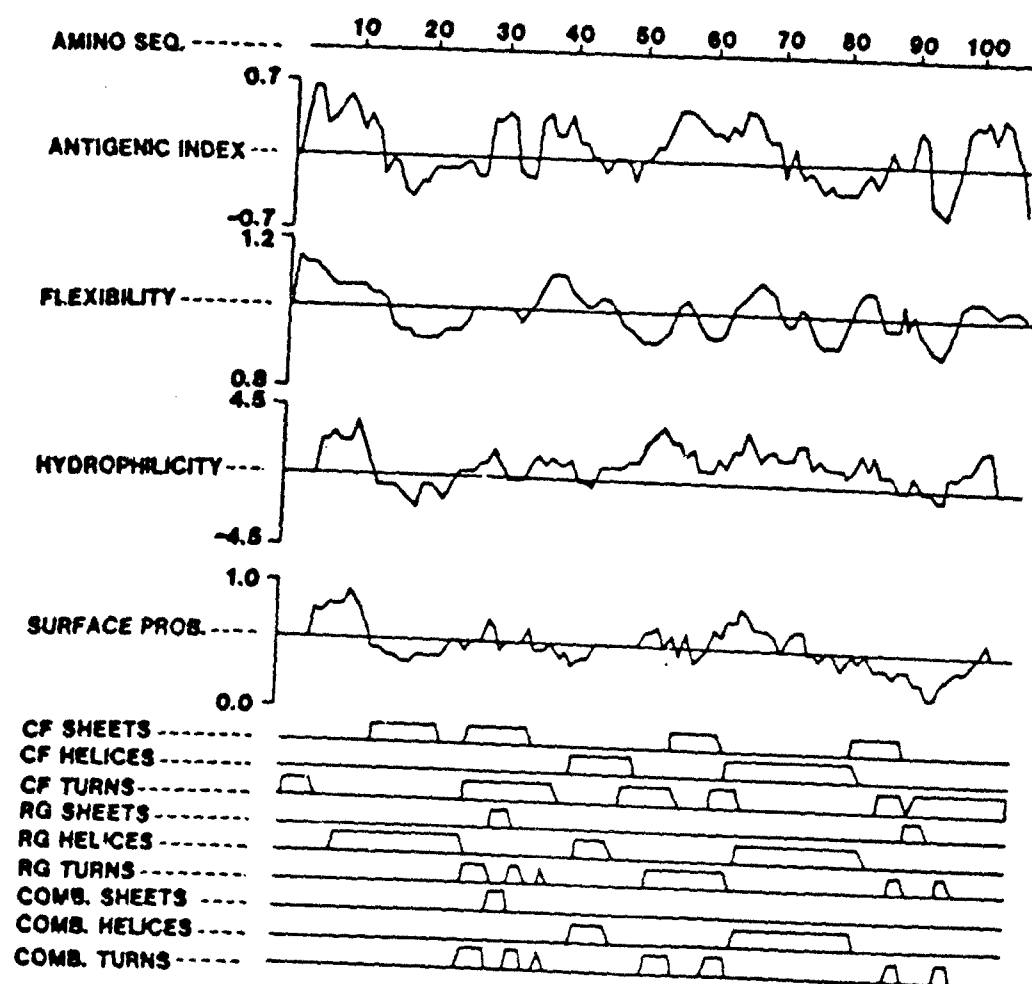


Figure 6. Antigenic index for toxin A peptide. The plots above were generated from the amino acid sequence of the toxin A peptide. The antigenic index was calculated from the hydrophilicity, surface probability, flexibility and secondary structure as described by Jameson and Wolf (28). Secondary structure predictions were performed according to the rules of Chou-Fasman (CF) (30), or Robson-Garnier (RG) (31). The secondary structure was also calculated based on a combination (COMB.) of Chou-Fasman and Robson-Garnier techniques.

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